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ORIGINAL RESEARCH ARTICLE

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Induction of protective immune response to intranasal administration of influenza virus-like particles in a mouse model

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Abstract

Human influenza A viruses (IAVs) cause global pandemics and epidemics, which remains a nonignorable serious concern for public health worldwide. To combat the surge of viral outbreaks, new treatments are urgently needed. Here, we design a new vaccine based on virus-like particles (VLPs) and show how intranasal administration of this vaccine triggers protective immunity, which can be exploited for the development of new therapies. H1N1 VLPs were produced in baculovirus vectors and were injected into BALB/c mice by the intramuscular (IM) or intranasal (IN) route. We found that there were significantly higher inflammatory cell and lymphocyte concentrations in bronchoalveolar lavage samples and the lungs of IN immunized mice; however, the IM group had little signs of inflammatory responses. On the basis of our results, immunization with H1N1 influenza VLP elicited a strong T cell immunity in BALB/c mice. Despite T cell immunity amplification after both IN and IM vaccination methods in mice, IN-induced T cell responses were significantly more intense than IM-induced responses, and this was likely related to an increased number of both $CD11b^{high}$ and $CD103^{+}$ dendritic cells in mice lungs after IN administration of VLP. Furthermore, evaluation of interleukin-4 and interferon gamma cytokines along with several chemokine receptors showed that VLP vaccination via IN and IM routes leads to a greater $CD4^{+}$ Th1 and Th2 response, respectively. Our findings indicated that VLPs represent a potential strategy for the development of an effective influenza vaccine; however, employing relevant routes for vaccination can be another important part of the universal influenza vaccine puzzle.

KEYWORDS
immunization, influenza A virus, therapy, virus-like particle (VLP)

1 | INTRODUCTION

Influenza viruses are responsible for global epidemics, causing 500 million infections—particularly in high-risk groups, such as the elderly—and between 250,000 and 500,000 deaths each year (Esghaei et al., 2018; Keshavarz, Dianat-Moghadam et al., 2018; Keshavarz, Tavakoli, Mozaffari Nejad, Mokhtari-Azad, & Rezaei, 2018; Shim, Kim, Tenson, Min, & Kainov, 2017). Currently, vaccination represents the most proficient strategy to prevent seasonal or pandemic influenza outbreaks (Control, C. f. D., & Prevention, 2013). Because seasonal vaccine requires annual reformulation on the basis of experiential predictions about the circulating virus strains in the coming year, it is essential to develop an effective influenza vaccine with a broad coverage against different strains and also for rapid production on a large scale with a low production cost (Fineberg, 2014).

Influenza virus-like particles (VLPs) are a new class of recombinant particulate vaccines comprised of hemagglutinin (HA), neuraminidase, matrix (M1), and ion channel (M2) proteins, which have been shown to produce bulk, safe, and low-cost influenza virus vaccines (Keshavarz, Mirzaei et al., 2018; Pushko et al., 2016; Wei et al., 2011). Because these vaccine platforms lack viral genome, they are considered attractive candidates for immunization in the elderly and young populations, groups that are at the highest risk of influenza complications (Quan et al., 2013). Influenza VLPs have many advantages because of their special structure, which is well uptaken and presented by the antigen-presenting cells and stimulate both effector and memory immune responses, it also has the ability to be used through an intramuscular (IM) and intranasal (IN) routes (Makarkov et al., 2017). Immunization with VLP through different routes, such as IN, IM, and parenteral, induces various aspects of adaptive and cellular immune responses (Perrone et al., 2009; Ren et al., 2018). VLP mucosal vaccination induces both local and systemic immunity, although the presence of appropriate adjuvants may lead to stronger immune responses (Malm, Tamminen, Vesikari, & Blazevic, 2015).

Seasonal influenza A virus initially shows tropism to the upper respiratory tract, whereas zoonotic viruses cause severe illness in the lower respiratory tract (Pizzolla et al., 2017) therefore IN immunization has an attractive route for defense against this pathogen (Luo et al., 2018).

Lung dendritic cells (LDCs) play a pivotal role in capturing and presenting antigens and thereby trigger immune responses. There are two subtypes of these cells in lung, which are capable of developing Th1 and Th2 responses, including CD103 and CD11b (Condon, Sawyer, Fenton, & Riches, 2011). When VLPs are delivered through the IN route, they are captured by LDCs and present via major histocompatibility complex (MHC) molecules to T cells. After the activation of LDCs, cytokines release and promote the expansion of CD8 and CD4 T cells (Si, Wen, Kelly, Chong, & Collier, 2018). The aim of this study was to assess the effect of H1N1 VLPs through muscular and intranasal immunization on lung dendritic cells (DCs) and on the major cytokines and chemokines that are inducted by Th1 and Th2 in mouse animal models.

2 | MATERIALS AND METHODS

2.1 | VLP production, purification, and characterization

H1N1 (PR8) VLPs containing H1, N1, and M1 were produced by using the recombinant baculoviruses (rBV) expression system by Rezaei et al. (Nadmdari, Keshavarz, Mokhtari-Azad, & Rezaei, 2017; Rezaei et al., 2013) and characterized by western blot and electron microscopy. For VLP verification, we used sodium dodecyl sulfate (SDS) 10–15% polyacrylamide gel and western blot analysis. For this, 3–5 ug of the purified VLP was loaded into the gel and was separated by SDS- polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane (Bio-Rad, CA). Subsequently, the membrane was blocked with 5% skim milk overnight at 4°C and then incubated by the primary H1, N1, and M1 mouse IgG mAbs (1:1,000 v/v) (Abcam, Cambridge, MA). After washing with Tris-buffered saline containing 0.1% Tween 20, the membrane was incubated with an HRP-conjugated secondary antibody (1:10,000 v/v) (Sigma-Aldrich, St. Louis, MO) for 2 hr at room temperature. Also for transmission electron microscopy, VLP specimens were adsorbed to the surface of carbon parlodion-coated copper grids (400 mesh) and stained with 1% phosphotungstic acid. The images of influenza VLP were recorded with a ZEISS EM900 transmission electron microscope at 80 kV with 85 kX magnification.

2.2 | Immunization

Female BALB/c mice, aged 6–8 weeks, were used in this study. A total of 12 female BALB/c mice in each group were intranasally (IN group) or intramuscularly (IM group) immunized with 10 ug H1N1 VLP. The control group (Mock) received only phosphate buffered saline (PBS). Thirty six hours after first immunization, three mice in each group were scarified and the lungs and bronchoalveolar lavage (BAL) fluids were collected for Haemotoxylin/Eosin (H&E) staining and cell counting, respectively. The mice received a booster dose seven days later, and Influenza virus-like particles then, 5 days after the second immunization, all mice were scarified with an intraperitoneal injection of 100 mg/kg ketamine. (All experiments were approved by and performed according to guidelines of the Animal Care and Use Committee from the Tehran University of Medical Sciences.)

2.3 | ELISA assay

Five days after second immunization with 10 ug H1N1 VLP, three mouse spleens were harvested from each groups, homogenized, and suspended in PBS. Red blood cells were lysed using Hemolytic Solution, and 10^5 splenocytes were seeded on 96-well plates. After ex-vivo restimulation by 4 ug/ml H1N1 VLPs for 24 hr at 37°C, suspensions were centrifuged and supernatants were collected. The enzyme-linked immunosorbent assay (ELISA) method was applied to measure cytokine responses as described by the manufacturer instructions of the Murine ELISA Development Kit (PeproTech Kits) to determine the amounts of interferon gamma (IFN-γ) and interleukin (IL)-4 cytokines.

2.4 | Real-time PCR

To evaluate immune biomarkers that were induced by H1N1 VLPs, total RNA was extracted from splenocyte after ex-vivo restimulation (as mentioned above) using TRIzol (Invitrogen, Carlsbad, CA) and converted to cDNA by a cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA). Real-time polymerase chain reaction (PCR) by specific primer sets and Universal PCR Master Mix System (Applied Biosystems) was used for relative quantification of target messenger RNA (mRNA). Table 1 indicates the primer sets for mouse mRNA targets (CXCR3, CXCR4, CCR4, and CCR5). All primers were designed to span exons and to avoid coamplification of genomic DNA. Specimens were tested and analyzed using the Step One Plus

TABLE 1 Primer sequences of target genes and reference gene used for relative quantitative Real-Time PCR

Target genes	Primer sequence
CXCR3	Forward: 5′-CAGCCTGAACTTTGACAGAACCT-3′ Reverse: 5′-GCAGCCCCAGCAAGAAGA-3′
CXCR4	Forward: 5′-TCAGCCTGGACCGGTACCT-3′ Reverse: 5′-GCAGTTTCCTTGGCCTTTGA-3′
CCR4	Forward: 5′-AGAAAATTCATGCGAGGGAGAA-3′ Reverse: 5′-TGTCACTCAAGGGTCGTGTCA-3′
CCR5	Forward: 5′-AGGCCATGCAGGCAACAG-3′ Reverse: 5′-TCTCTCCAACAAAGGCATAGATGA-3′
β-actin	Forward: 5′-GCTCTGGCTCCTAGCACCAT-3′ Reverse: 5′-GCCACCGATCCACACAGAGT-3′

Note. PCR: polymerase chain reaction.

Real-Time PCR System (Applied Biosystems) and LinRegPCR (www.hartfaalcentrum.nl), respectively. Ct values were normalized to β-actin mRNA and expressed as the fold change relative to the mean Ct value for the control samples using the Ct method.

2.5 | Bronchoalveolar lavage cells' counting, histology, and flow cytometry

Thirty-six hours after the first immunization, BAL fluids were collected from the thoracic cavity ofanesthetized anesthetized BALB/c mice by washing the lung with 2 ml of sterile cold PBS. After that, BAL fluid was centrifuged at 300*g* for 10 min, and pellet cells were stained with Giemsa stain. Each slide was counted three times under optical microscope, and the percentage of leukocytes was calculated according to the morphological criteria. Also, from each group three mice were scarified, and lung sections were prepared and fixed for H&E staining as described by Baligar, Pokhrel, and Mukhopadhyay (2016). Finally, the slides were evaluated by an optical microscope.

The efficacy of lung-proliferated T and DC cells were determined by flow cytometry as described (Song et al., 2011; Ghaedi et al., 2015). Briefly, the homogenized lung tissues were treated with 100 μg per ml digestion buffer (DNase I) for 30 min at 37°C and then passed through a cell strainer (40 mm, BD Diagnostics, Franklin Lakes, NJ). The remaining single-cell suspensions were stained with fluorescence-conjugated antibodies specific to cell phenotypes (CD4, CD8, CD103, MHCII, CD11c, and CD11b). The lung DC cells were gated according to their sizes and granularity defined in the forward light scatter and side light scatter plot and sorted on the basis of their CD4/CD8, CD11c/MHCII, and CD11b/CD103 profiles. Cell acquisition was performed with a dual-laser flow cytometer (Attune NxT flow cytometer, Foster City, CA), and data were analyzed using the FlowJo software (Tree Star Inc., San Carlos, CA).

2.6 | Statistical analysis

The expression as fold change related to immune responses was evaluated according to the Ct method. The average value and standard deviation for the level of immune responses within the treated and control group were calculated for comparison, and the significance of the differences between the results from different groups was determined by Student's *t* test using the GraphPad Prism software (V7.0). *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$ were considered statistically significant.

3 | RESULTS

3.1 | Expression and characterization of proteins

To produce VLP, the recombinant-rBV-expressing influenza H1, N1, and M1 was inoculated into Sf9 cells, as described by Rezaei et al. (2013). Three days after transfection, cell lysates were subjected to SDS, and western blot analysis showed the presence of the H1, N1, and M1 proteins in the cell lysate. H1, N1, and M1 proteins expressed in Sf9 cells represented a polypeptide of approximately 63, 50, and 29 kDa, as expected (Figure 1a). In addition, the images of the electron microscope showed pleomorphic VLPs of sizes ranging from 80 to 120 nm (Figure 1b,c).

3.2 | Cells distribution in mouse BAL fluid

To assess the immune cell mixture in the lung mucus of BALB/c mice, BAL samples were collected from the pulmonary cavity by washing the lungs with 2 ml of sterile PBS. After centrifugation at 300 g for 10 min, Giemsa staining was used to evaluate the pellet cells. By counting the stained slides, we found a significantly higher abundance of lymphocytes in BAL fluid of IN immunized mice than IM and mock groups (Figure 2a). The concentration of monocytes also was significantly greater in the BAL samples of IN immunized mice (Figure 2b). Similarly, a number of infiltrated neutrophils showed a significant increase in the BAL samples of IN immunized mice (Figure 2c). Overall, the BAL samples of IN vaccinated mice had a significantly greater total immune cell content than the two other groups (Figure 2d). These data suggest a remarkable inflammatory and lymphocyte response in lung mucus after immunization through the IN route.

3.3 | Chemokine receptor expression

We evaluated the chemokine receptor expression in the harvested spleens from IN or IM vaccinated and nonvaccinated mice. After RNA extraction from the lysates of spleen cells and cDNA synthesis, the mRNA expression level of four chemokine receptors was measured by real-time PCR using specific primer sets. The results of real-time PCR showed that after IN VLP immunization, CXCR3 and CCR5 chemokine receptors of T cells had a significantly higher mRNA expression level than those in the IM or mock groups. On the other hand, contrary to those in the IN and the mock groups, the mRNA expression levels of CXCR4 and CCR4 chemokine receptors were significantly higher in the IM group (Figure 3).

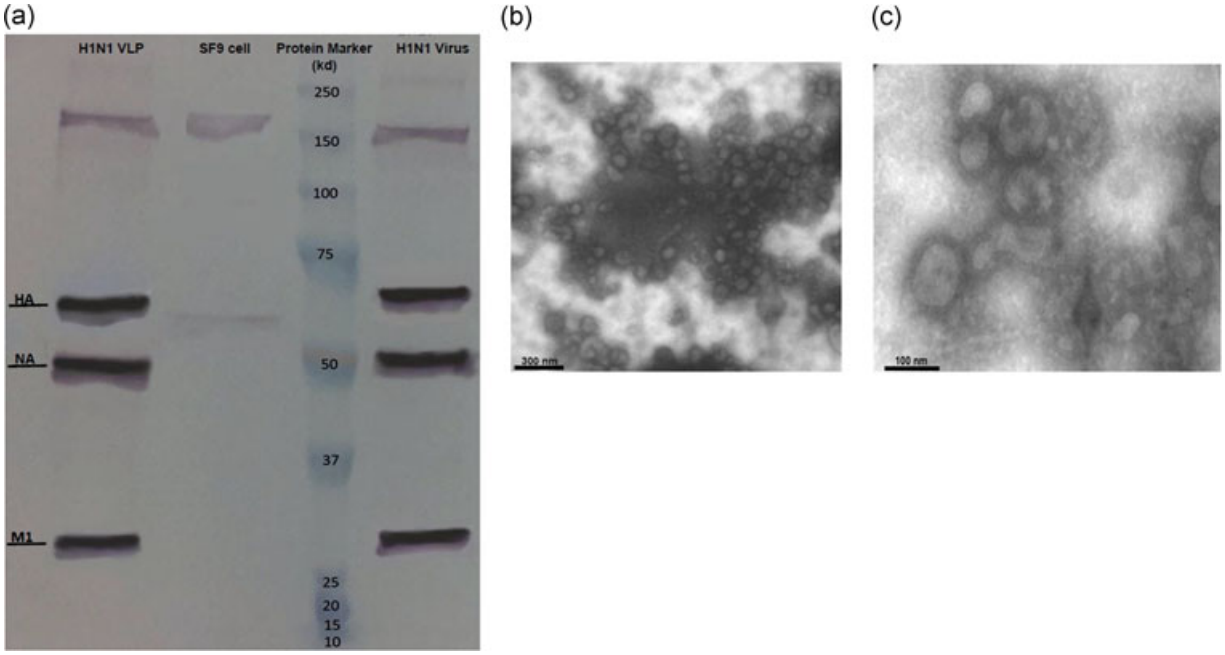

FIGURE 1 Characterization of influenza H1N1 VLP. Western blot analysis of H1N1 VLP produced in sf9 cells with anti-H1, N1, and M1 Mabs, Sf9 cell, and H1N1 virus were used as negative and positive control, respectively. The molecular weight of H1, N1, and M1 proteins were 63, 50, and 29 KDa, respectively (a). Negative staining with 1% phosphotungstic acid and transmission electron microscopy shows influenza VLPs (b,c). Bar represents 300 and 100 nm, respectively. VLP: virus-like particle [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | Cytokine responses

Then, we compared the cytokine response of splenocytes obtained from each group of vaccinated and nonvaccinated mice in this study. After ex-vivo restimulation of splenocytes with the same VLP, the ELISA method was utilized to measure the quantity of IFN-γ and IL-4 cytokines in the supernatants collected from splenocytes suspensions. On the basis of the data of this section, IN immunization led to a significant increase of the IFN-γ level in splenocytes (Figure 4a). In terms of IL-4, although IM immunization induced a higher IL-4 response than IN immunization, it was not significant. However, the level of IL-4 in both IM and IN groups had a significant increase compared with the mock group (Figure 4b). These results were indicative of almost opposite cytokine response patterns in IN and IM immunized mice.

3.5 | Lung immune cell responses and variation

To determine the rate of immune cell recruitment in lungs after immunization, we examined the lung tissue sections of mice. After sacrificing the mice, lungs were harvested and sections were prepared, fixed, and finally stained with H&E to be evaluated under an optical microscope. We observed a high frequency of lung-tissue-infiltrating immune cells in the IN immunized group (Figure 5a). On the other hand, the lung sections of the IM immunized group (Figure 5b) and the mock group (Figure 5c) had no evident infiltration of immune cells. The results obtained from the IN group in this part indicated an intense concentration of immune cells in lung tissues in response to mucosal immunization and were consistent with the results of the cell distribution in BAL samples.

3.6 | T cell and dendritic cell responses

The homogenized lung samples were treated with digestion buffer. Single-cell suspensions were stained with fluorescence-conjugated antibodies specific to cell phenotypes. The lung DC cells were gated according to their sizes and granularity and sorted on the basis of their CD4/CD8, CD11c/MHCII, and CD11b/CD103 profiles. Cell acquisition was performed with a dual-laser flow cytometer, and data were analyzed using the FlowJo software. According to our data, after IN administration of VLP, there is a significant increase in the number of both $CD11b^{high}$ and $CD103^{+}$ DCs in lung samples, whereas after receiving IM VLP or PBS (mock), the population of DCs showed no significant changes. Our findings also demonstrated that there is a significant increase in $CD4^{+}$ and $CD8^{+}$ cellular responses in IN-VLP-immunized mice in comparison with IM vaccinated and control groups (Figure 6).

4 | DISCUSSION

In the present study, we investigated the efficacy of T-cell immune responses after IN or IM administration of influenza VLP vaccines in BALB/c mice and compared the immune response patterns after immunization through the mentioned routes. The H1N1 VLP vaccine utilized in this study was developed by using a recombinant baculovirus-based expression of H1, N1, and M1 proteins in the Sf9 cell line, which has been proved to be a safe, high-yield, and low-cost vaccine production method (Bright et al., 2008). It has been shown that VLPs, as new vaccine candidates, can be produced with a high yield in a short time period and are also promising platforms to enhance the

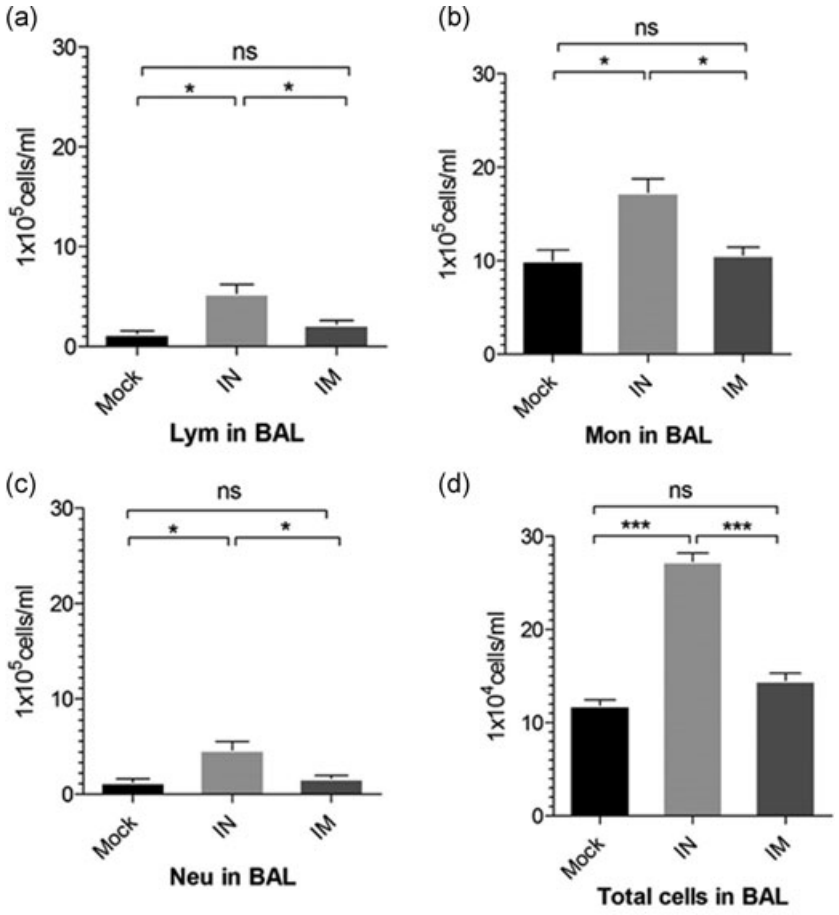

FIGURE 2 The distribution of immune cells in BAL fluid of mice immunized IM and IN with H1N1 VLP. Immune cells were collected from BAL fluid 36 hr post immunization. The numbers of lymphocytes (Lym; a), monocytes (Mon; b), neutrophils (Neu; c), and total cell content (d) were analyzed in the BAL fluid. A remarkable inflammatory and lymphocyte response in lung mucus after immunization through the IN route (a–d). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). BAL: bronchoalveolar lavage; IM: intramuscular; IN: intranasal

immunogenicity of antigens (Bright et al., 2007; Fries, Smith, & Glenn, 2013; Hossain et al., 2011; Wang et al., 2012). Because of their multivalent particulate structure, VLPs can be taken up and processed by dendritic cells (DCs) more efficiently and, thereupon, can strongly provoke effector humoral and cellular immune responses, making them more immunogenic than other vaccine types (Chackerian, Lenz, Lowy, & Schiller, 2002; Harper et al., 2006; Haynes, 2009; Lenz et al., 2001; Lenz et al., 2003; Song et al., 2010). In addition, VLPs are safer vaccine products compared with the former traditional live attenuated or inactivated vaccines (Roldão, Mellado, Castilho, Carrondo, & Alves, 2010). Despite many advantages, VLP vaccines produced on the basis of insect cells are also (Sf9) subject to certain restrictions. One of the important attributes of these cells is contamination with baculovirus particles (similar densities with influenza VLP) or baculovirus DNA. Indeed, this contamination induces immune responses and produces unwanted cytokines responses. On the other hand, production and purification of some VLPs may represent a limitation, especially for enveloped recombinant virus-like particles (rVLPs; Margine, Martinez-Gil, Chou, & Krammer, 2012; Pitoiset, Vazquez, & Bellier, 2015).

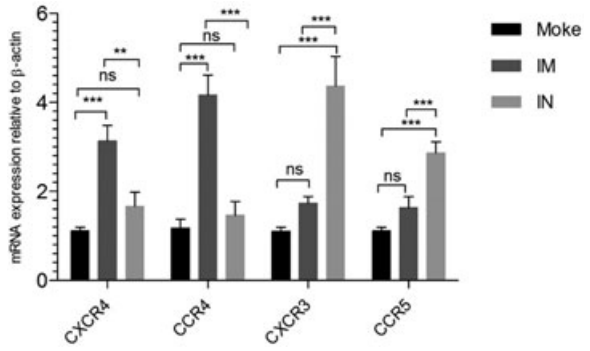

FIGURE 3 mRNA expression levels of four chemokine receptor genes in splenocytes after ex-vivo restimulation by 4 ug/mlH1N1 VLPs. CXCR3 and CCR5 chemokine receptors of T cells had a significantly higher mRNA expression level in the IN group, whereas CXCR4 and CCR4 showed an increase in IM immunized mice. (**$p < 0.01$, ***$p < 0.001$). IN: intranasal; mRNA: messenger RNA; VLP: virus-like particle

The VLP vaccine described in this study also showed considerable immunogenicity and stimulated protective cellular immune responses as well.

A growing body of evidence emphasizes the determinative effect of administration route on the immunization outcome. A majority of the current research on VLP administration ahs focused their work on IN and IM routes (Hodgins et al., 2017; Price, Lo, Misplon, & Epstein, 2018; Ren et al., 2018; Trondsen et al., 2015), although other ways, such as the intradermal route, are under investigation (Quan et al., 2010). A critical requirement for comparing these routes usually includes a magnitude of stimulated inflammatory as well as protective immune responses. Here, we evaluated some immunological aspects of IN and IM immunization of mice by VLPs.

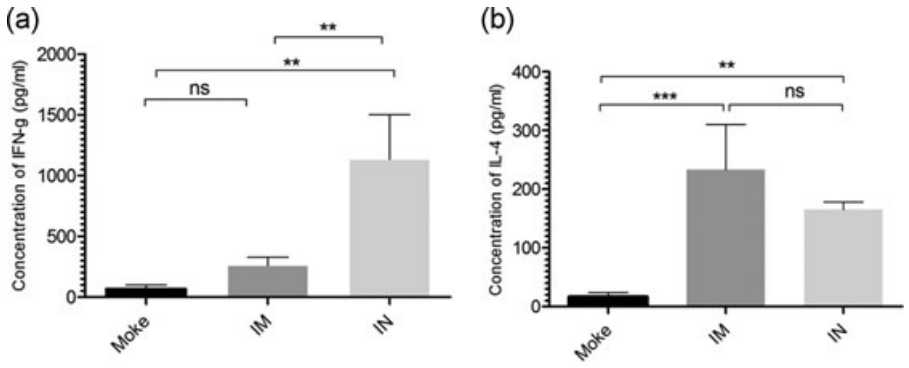

FIGURE 4 The concentration of two cytokines produced by splenocytes after ex vivo stimulation. Five days after the second immunization, splenocytes were collected and stimulated ex vivo for 24 hr with 4 ug/ml H1N1 VLP. Concentrations of IFN-γ (a) and IL-4 cytokines (b) in the supernatant were measured by ELISA. There were two different cytokine response patterns after IN and IM immunization. (a,b). (**$p < 0.01$, ***$p < 0.001$). ELISA: enzyme-linked immunosorbent assay; IFN-γ: interferon; IL: interleukin; IM: intramuscular; IN: intranasal; mRNA: messenger RNA; VLP: virus-like particle

IN immunization with VLPs can alter the immune cell content of lung mucus and density of cell infiltration within the lung tissue in mice (Ren et al., 2018). Our results imply a significantly higher concentration of inflammatory cells (i.e. monocytes and neutrophils) and lymphocytes in the BAL samples of IN vaccinated mice compared with IM and mock groups. In the same way, we found a large number of infiltrated immune cells in the lung tissues of IN immunized mice, but there were no signs of accumulation of immune cells in the lungs of IM immunized or mock groups. The plain reason for these increased cellular and inflammatory responses in BAL and lung, in addition to mucosal stimulation provided via the IN route, is probably the partial imitation of the immune response pattern of influenza infection by the VLPs. According to our survey, VLP administration can induce an almost considerable inflammatory response. However, as shown recently, immunizing mice with VLPs before influenza infection leads to a significant decrease in inflammatory cells in BAL and lungs compared with nonimmunized, influenza-infected mice (Kim et al., 2015; Lee et al., 2016; Lee et al., 2018).

As mentioned above, VLPs can be taken up by DCs more efficiently, leading to a stronger immune response. Sung et al. (2006) have reported that mouse LDCs possess two major subsets, including $CD11c^+$ MHC class II^+ CD11 blow or negative $CD103^+$ LDCs ($CD103^+$ LDCs) and $CD11c^+$ MHC class II^+ $CD11b^{high}$ $CD103^-$ LDCs ($CD11b^{high}$ LDCs). A study highlights that mice $CD103^+$ LDCs induce proliferation of $CD4^+$ T cells expressing higher levels of IFN-γ and also CXCR3 and CCR5 chemokine receptors, indicating a dominant Th1 response, whereas induction by $CD11b^{high}$ LDCs mediates proliferation of $CD4^+$ T cells expressing higher levels of IL-4, CXCR4, and CCR4, exhibiting a greater Th2 response (Furuhashi et al., 2012). According to our data, after IN administration of VLP, a significant increase occurred in the number of both $CD11b^{high}$ and $CD103^+$ DCs in mice lung, whereas after receiving IM VLP, the population of DCs showed no significant change. Because $CD11b^{high}$ and $CD103^+$ DCs are lung-resident cells, IN vaccination as a principal stimulant of mucosal immunity, raises the frequency of both DCs in mice lungs, and this fact can be indicative of a propensity toward approximately balanced Th1 and Th2 cellular responses. This was after IN VLP immunization, CXCR3 and CCR5 chemokine receptors of $CD4^+$ T cells in spleens had a higher mRNA expression level than those in IM or mock groups; however, the CXCR4 and CCR4 expression levels were higher in the IM group than IN and mock groups, representing greater $CD4^+$ Th1 and Th2 responses after IN and IM immunization with VLP, respectively.

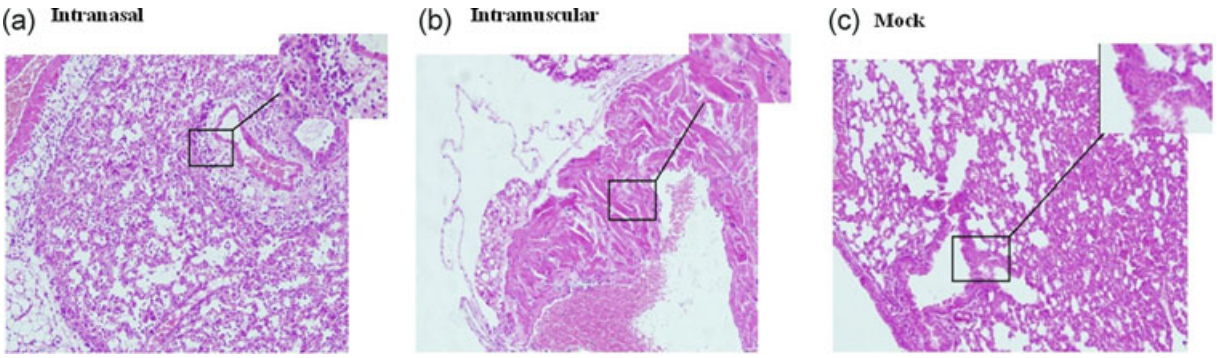

FIGURE 5 The lungs were removed from BALB/c mice 36 hr after immunization with H1N1 VLPs. (a) The lungs had increased cell population as triggered by the infiltration of immune cells and triggered the APC process in the IN route. (b,c) The lung tissue from IM immunized and control groups (Mock) did not show significant cells infiltration. H&E staining colors cytoplasm pink and nuclei blue. APC: antigen-presenting cell; IM: intramuscular; IN: intranasal; H&E: hemotoxylin and eosin; VLP: virus-like particle [Color figure can be viewed at wileyonlinelibrary.com]

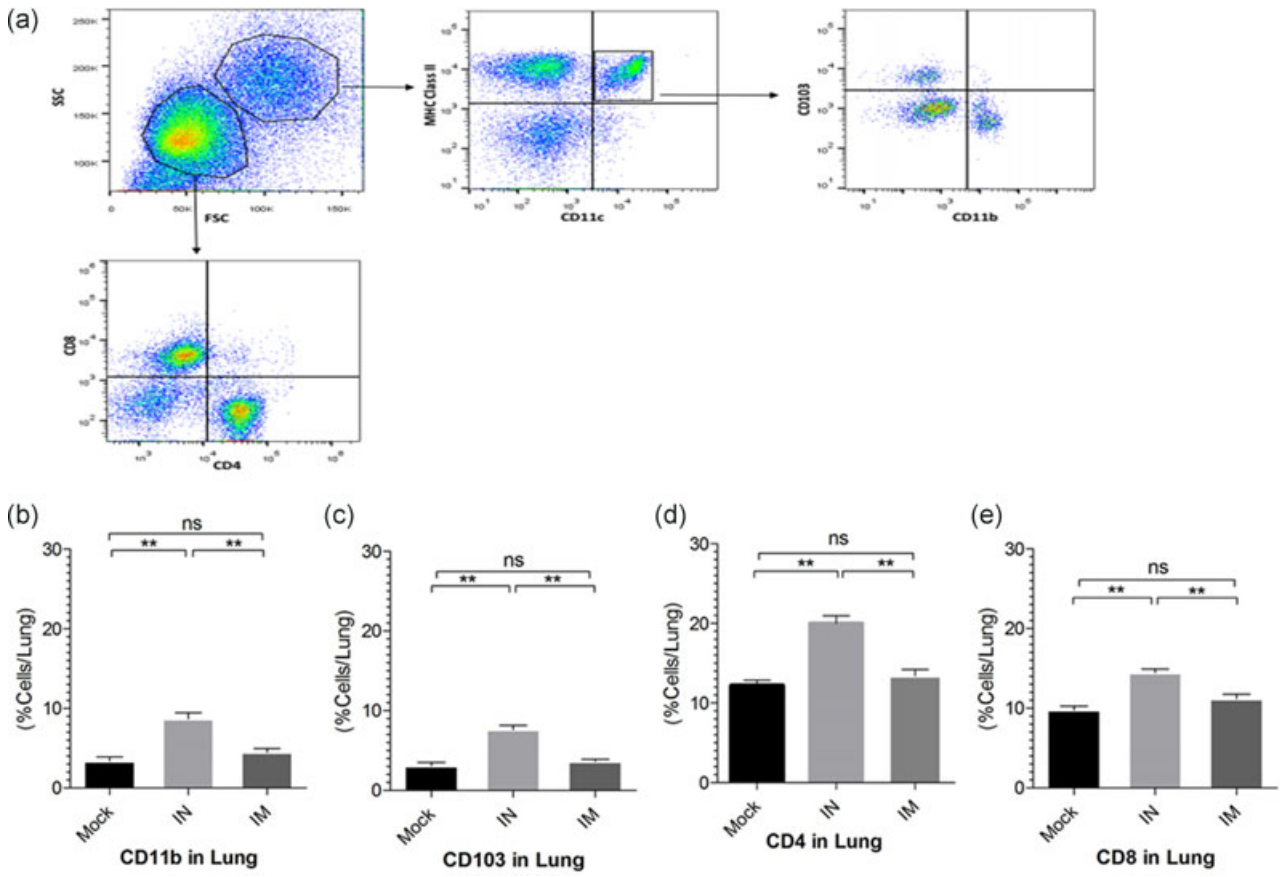

FIGURE 6 The percentage of T cells and dendritic cells in lung tissue. IN immunized mice induced $CD103^+$ and $CD11b^+$ DCs in the lungs (a–e). Percentage of $CD11b^+$ and $CD103^+$ DC populations gated from $CD11c^+$ $MHCII^+$. Significant induction of $CD4^+$ and $CD8^+$ cells in the lung was seen in the IN group than IM immunized or control groups (a–e). (**$p < 0.01$). DC: dendritic cell; IM: intramuscular; IN: intranasal [Color figure can be viewed at wileyonlinelibrary.com]

Similarly, we showed that IN immunization leads to a considerable increase in the IFN-γ level in splenocytes, indicating a significant trend toward $CD4^+$ Th1 response, which was comparable with the chemokine receptor results. Although IL-4 production induced by IM immunization was slightly higher compared with that of IN immunization—which suggests a somewhat higher Th2 response—the difference was not significant. Several other studies also have emphasized that measuring cytokine and chemokine levels in spleen and lung can be very helpful in determining the type and efficacy of immune responses after immunization and have shown that different cytokine responses can be caused by immunization from IN or IM routes (Hodgins et al., 2017; Hu et al., 2017; Kim et al., 2013; Trondsen et al., 2015). In a study of Hodgins et al. (2017), the levels of Th1-inducing cytokines were higher in the spleens of IN immunized mice, but the IM immunized ones had greater levels of Th2-related cytokines. Another survey by VLPs expressed that more quantities of IFN-γ were detected in the splenocytes of IN vaccinated mice, but the levels of IL-4 were almost equal in both IM and IN immunized sets. Also, Trondsen et al. (2015) reported results similar to our study in terms of Th1 and Th2 specific cytokines by using another vaccine platform.

On the basis of the available evidence, $CD4^+$ and $CD8^+$ T cells have an undeniable role in recovery from influenza infection (Lee et al., 2018), and interestingly, after influenza VLP immunization, a remarkable increase occurs in cross-reactive poly-functional T cell populations in lungs (Landry et al., 2014). On the basis of previous studies, different vaccine administration routes have various activation results in terms of T cell responses (Hemann, Kang, & Legge, 2013; Landry et al., 2014; Pillet et al., 2016; Sung et al., 2006). By comparing the studied groups, we demonstrated that contrary to IM immunized or control groups, IN VLP immunized mice develop significantly more intense $CD4^+$ and $CD8^+$ cellular responses in the lungs. Regarding the presence of high frequencies of specific DCs in lungs, such increases in the population of T cells are explainable and can be confirmed by obvious infiltration of lymphocytes in BAL and lung sections. Ren et al. (2018) reported that although IM vaccination by H7N9 VLPs causes elevated levels of the lung, existing $CD4^+$ and $CD8^+$ memory cells, VLPs administered IN, inducing a stronger response of this type. In a former study on whole inactivated influenza virus vaccine, $CD4^+$ cells with various functionalities were detected in higher numbers in the IN than IM immunized group of mice (Trondsen et al., 2015). During a clinical trial study, IM injection of H1 and H5 VLP vaccines provoked greater polyfunctional $CD4^+$ and $CD8^+$ T-cell responses than trivalent inactivated vaccine and placebo, which

were durable for more than 6 months (Landry et al., 2014). Also, as Hemann et al. (2013) demonstrated, IN vaccination with influenza VLPs leads to a considerable increase in HA-specific CD8 T cells in mice lungs. In light of what is explained here, vaccination with VLP can be an effective factor in limiting and clearing the infection in the lungs through enhancing the response of various T-cell lines. Of course, as discussed above, lung habitant DCs play an essential role in triggering such functional T cell responses.

5 | CONCLUSION

We have shown that immunization with H1N1 influenza VLP elicits a strong and protective T cell immunity in BALB/c mice. We also demonstrated that vaccination from both IN and IM routes leads to augmenting T-cell immunity in mice however, IN induced T-cell responses were significantly more extensive than those induced through the IM route, which was probably because of the increased frequency of $CD103^{+}$ and $CD11b^{high}$ DCs in the lungs. On the other hand, IN vaccination led to a $CD4^{+}$ T-cell response trend towards Th1, whereas a greater Th2 response was motivated via IM route. These data suggest that VLPs could be introduced as stimulators of cellular and humoral immune responses in comparison with their previous counterparts, which, along with better safety profile reported in other studies, can reflect the clear superiority of VLP vaccines. Moreover, this study characterized the IN route as a more advantageous vaccination method. Taken together, the results of this study, as well as other similar studies, illustrate that selecting an appropriate route for vaccination is an effective factor on immunization result. Accordingly, more comparative preclinical and clinical surveys are needed to specify the most reasonable route or a combination of routes for VLP vaccine administration in humans.

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CONFLICT OF INTEREST

The authors declare there are no conflict of interests.

AUTHOR CONTRIBUTIONS

F. R. and T. M. contributed in conception, design, statistical analysis, and drafting of the manuscript. M. K., H. N., Y. A., H. M., V. S., A. S., and T. M. contributed in data collection and manuscript drafting. All authors approved the final version for submission.

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REFERENCES

Baligar, P., Pokhrel, S., & Mukhopadhyay, A. (2016). Experimental liver fibrosis and intrasplenic transplantation of CD45+ bone marrow cells. *Bio-protocol*, *6*(20) https://doi.org/10.21769/BioProtoc.1972

Bright, R. A., Carter, D. M., Crevar, C. J., Toapanta, F. R., Steckbeck, J. D., Cole, K. S., … Ross, T. M. (2008). Cross-clade protective immune responses to influenza viruses with H5N1 HA and NA elicited by an influenza virus-like particle. *PLoS One*, *3*(1), e1501. https://doi.org/10.1371/journal.pone.0001501

Bright, R. A., Carter, D. M., Daniluk, S., Toapanta, F. R., Ahmad, A., Gavrilov, V., … Ross, T. M. (2007). Influenza virus-like particles elicit broader immune responses than whole virion inactivated influenza virus or recombinant hemagglutinin. *Vaccine*, *25*(19), 3871–3878. https://doi.org/10.1016/j.vaccine.2007.01.106

Chackerian, B., Lenz, P., Lowy, D. R., & Schiller, J. T. (2002). Determinants of autoantibody induction by conjugated papillomavirus virus-like particles. *Journal of Immunology*, *169*(11), 6120–6126.

Condon, T. V., Sawyer, R. T., Fenton, M. J., & Riches, D. W. H. (2011). Lung dendritic cells at the innate-adaptive immune interface. *Journal of Leukocyte Biology*, *90*(5), 883–895. https://doi.org/10.1189/jlb.0311134

Control, C. F. D., & Prevention (2013). Estimated influenza illnesses and hospitalizations averted by influenza vaccination-United States, 2012-13 influenza season. *MMWR. Morbidity and Mortality Weekly Report*, *62*(49), 997–1003.

Esghaei, M., Moghoofei, M., Keshavarz, M., Keyvani, H., Bokharaei-Salim, F., Farahmand, M., & Monavari, S. H. (2018). Trends in surveillance data of influenza virus in Tehran before decreasing dispatch of Iranian Hajj pilgrims to Mecca. *Medical Journal of the Islamic Republic of Iran*, *32*, 41–238. https://doi.org/10.14196/mjiri.32.41

Fineberg, H. V. (2014). Pandemic preparedness and response—lessons from the H1N1 influenza of 2009. *New England Journal of Medicine*, *370*(14), 1335–1342.

Fries, L. F., Smith, G. E., & Glenn, G. M. (2013). A recombinant viruslike particle influenza A (H7N9) vaccine. *New England Journal of Medicine*, *369*(26), 2564–2566. https://doi.org/10.1056/NEJMc1313186

Furuhashi, K., Suda, T., Hasegawa, H., Suzuki, Y., Hashimoto, D., Enomoto, N., … Chida, K. (2012). Mouse lung $CD103^{+}$and $CD11b^{high}$ dendritic cells preferentially induce distinct $CD4^{+}$ T-cell responses. *American Journal of Respiratory Cell and Molecular Biology*, *46*(2), 165–172. https://doi.org/10.1165/rcmb.2011-0070OC

Ghaedi, M., Namdari, H., Rahimzadeh, P., Mortezagholi, S., Azimi-Mohamadabadi, M., … Salehi, E. (2015). Different doses of transforming growth factor-β on in vitro differentiation of human naïve CD4+ T cells to T Helper 17. *Iranian Journal of Allergy, Asthma and Immunology*, *14*(6), 633–637.

Harper, D. M., Franco, E. L., Wheeler, C. M., Moscicki, A. B., Romanowski, B., Roteli-Martins, C. M., … Dubin, G. (2006). Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: Follow-up from a randomised control trial. *Lancet*, *367*(9518), 1247–1255. https://doi.org/10.1016/s0140-6736(06)68439-0

Haynes, J. R. (2009). Influenza virus-like particle vaccines. *Expert Review of Vaccines*, *8*(4), 435–445. https://doi.org/10.1586/erv.09.8

Hemann, E. A., Kang, S. M., & Legge, K. L. (2013). Protective CD8 T cell-mediated immunity against influenza A virus infection following influenza virus-like particle vaccination. *Journal of Immunology*, *191*(5), 2486–2494. https://doi.org/10.4049/jimmunol.1300954

Hodgins, B., Yam, K. K., Winter, K., Pillet, S., Landry, N., & Ward, B. J. (2017). A single intramuscular dose of a plant-made virus-like particle vaccine elicits a balanced humoral and cellular response and protects young and aged mice from influenza H1N1 virus challenge despite a modest/absent humoral response. *Clinical and Vaccine Immunology*, *24*(12), e00273–17. https://doi.org/10.1128/cvi.00273-17

Hossain, M. J., Bourgeois, M., Quan, F. S., Lipatov, A. S., Song, J. M., Chen, L. M., … Donis, R. O. (2011). Virus-like particle vaccine containing hemagglutinin confers protection against 2009 H1N1 pandemic influenza. *Clinical and Vaccine Immunology*, *18*(12), 2010–2017. https://doi.org/10.1128/cvi.05206-11

Hu, C. M. J., Chien, C. Y., Liu, M. T., Fang, Z. S., Chang, S. Y., Juang, R. H., … Chen, H. W. (2017). Multi-antigen avian influenza a (H7N9) virus-like particles: Particulate characterizations and immunogenicity evaluation in murine and avian models. *BMC Biotechnology*, *17*(1), 2. https://doi.org/10.1186/s12896-016-0321-6

Keshavarz, M., Dianat-Moghadam, H., Sofiani, V. H., Karimzadeh, M., Zargar, M., Moghoofei, M., … Mirzaei, H. (2018). miRNA-based strategy for modulation of influenza A virus infection. *Epigenomics*, *10*(6), 829–844. https://doi.org/10.2217/epi-2017-0170

Keshavarz, M., Mirzaei, H., Salemi, M., Momeni, F., Mousavi, M. J., Sadeghalvad, M., … Rezaei, F. (2018). Influenza vaccine: Where are we and where do we go? *Reviews in Medical Virology*, *29*, e2014. https://doi.org/10.1002/rmv.2014

Keshavarz, M., Tavakoli, A., Mozaffari Nejad, A. S., Mokhtari-Azad, T., & Rezaei, F. (2018). A review of influenza vaccination among different population groups in Iran. *Journal of Clinical & Diagnostic Research*, *12*(6), LE09–LE13.

Kim, K. H., Lee, Y. T., Hwang, H. S., Kwon, Y. M., Kim, M. C., Ko, E. J., … Kang, S. M. (2015). Virus-like particle vaccine containing the F protein of respiratory syncytial virus confers protection without pulmonary disease by modulating specific subsets of dendritic cells and effector T cells. *Journal of Virology*, *89*(22), 11692–11705. https://doi.org/10.1128/jvi.02018-15

Kim, M. C., Song, J. M., O, O., Kwon, Y. M., Lee, Y. J., Compans, R. W., & Kang, S. M. (2013). Virus-like particles containing multiple M2 extracellular domains confer improved cross-protection against various subtypes of influenza virus. *Molecular Therapy*, *21*(2), 485–492. https://doi.org/10.1038/mt.2012.246

Landry, N., Pillet, S., Favre, D., Poulin, J. F., Trépanier, S., Yassine-Diab, B., & Ward, B. J. (2014). Influenza virus-like particle vaccines made in Nicotiana benthamiana elicit durable, poly-functional and cross-reactive T cell responses to influenza HA antigens. *Clinical Immunology*, *154*(2), 164–177. https://doi.org/10.1016/j.clim.2014.08.003

Lee, Y. T., Ko, E. J., Lee, Y., Kim, K. H., Kim, M. C., Lee, Y. N., & Kang, S. M. (2018). Intranasal vaccination with M2e5x virus-like particles induces humoral and cellular immune responses conferring cross-protection against heterosubtypic influenza viruses. *PLoS ONE*, *13*(1), e0190868. https://doi.org/10.1371/journal.pone.0190868

Lee, Y. T., Ko, E. J., Lee, Y., Lee, Y. N., Bian, Z., Liu, Y., & Kang, S. M. (2016). CD47 plays a role as a negative regulator in inducing protective immune responses to vaccination against influenza virus. *Journal of Virology*, *90*(15), 6746–6758. https://doi.org/10.1128/jvi.00605-16

Lenz, P., Day, P. M., Pang, Y. Y. S., Frye, S. A., Jensen, P. N., Lowy, D. R., & Schiller, J. T. (2001). Papillomavirus-like particles induce acute activation of dendritic cells. *Journal of Immunology*, *166*(9), 5346–5355.

Lenz, P., Thompson, C. D., Day, P. M., Bacot, S. M., Lowy, D. R., & Schiller, J. T. (2003). Interaction of papillomavirus virus-like particles with human myeloid antigen-presenting cells. *Clinical Immunology*, *106*(3), 231–237.

Luo, J., Lu, G., Ye, S., Ou, J., Fu, C., Zhang, X., … Li, S. (2018). Comparative pathogenesis of H3N2 canine influenza virus in beagle dogs challenged by intranasal and intratracheal inoculation. *Virus Research*, *255*, 147–153.

Makarkov, A. I., Chierzi, S., Pillet, S., Murai, K. K., Landry, N., & Ward, B. J. (2017). Plant-made virus-like particles bearing influenza hemagglutinin (HA) recapitulate early interactions of native influenza virions with human monocytes/macrophages. *Vaccine*, *35*(35), 4629–4636.

Malm, M., Tamminen, K., Vesikari, T., & Blazevic, V. (2015). Comparison of intramuscular, intranasal and combined administration of norovirus virus-like particle subunit vaccine candidate for induction of protective immune responses in mice.

Margine, I., Martinez-Gil, L., Chou, Y., & Krammer, F. (2012). Residual baculovirus in insect cell-derived influenza virus-like particle preparations enhances immunogenicity. *PLoS One*, *7*(12), e51559.

Nadmdari, H., Keshavarz, M., Mokhtari-Azad, T., & Rezaei, F. (2017). Evaluation of antibody and cytokines responses in intranasally and intramuscularly administrated BALB/C mice with influenza virus-like particle. *Acta Medica Iranica*, *55*(10), 604–611.

Perrone, L. A., Ahmad, A., Veguilla, V., Lu, X., Smith, G., Katz, J. M., … Tumpey, T. M. (2009). Intranasal vaccination with 1918 influenza virus-like particles protects mice and ferrets from lethal 1918 and H5N1 influenza virus challenge. *Journal of Virology*, *83*(11), 5726–5734.

Pillet, S., Aubin, É., Trépanier, S., Bussière, D., Dargis, M., Poulin, J. F., … Landry, N. (2016). A plant-derived quadrivalent virus like particle influenza vaccine induces cross-reactive antibody and T cell response in healthy adults. *Clinical Immunology*, *168*, 72–87. https://doi.org/10.1016/j.clim.2016.03.008

Pitoiset, F., Vazquez, T., & Bellier, B. (2015). Enveloped virus-like particle platforms: Vaccines of the future? *Expert Review of Vaccines*, *14*(2), 1–3.

Pizzolla, A., Nguyen, T. H. O., Smith, J. M., Brooks, A. G., Kedzierska, K., Heath, W. R., … Wakim, L. M. (2017). Resident memory $CD8^+$T cells in the upper respiratory tract prevent pulmonary influenza virus infection. *Science immunology*, *2*(12), eaam6970. https://doi.org/10.1126/sciimmunol.aam6970

Price, G. E., Lo, C. Y., Misplon, J. A., & Epstein, S. L. (2018). Reduction of influenza virus transmission from mice immunized against conserved viral antigens is influenced by route of immunization and choice of vaccine antigen. *Vaccine*, *36*(32 Pt B), 4910–4918. https://doi.org/10.1016/j.vaccine.2018.06.051

Pushko, P., Sun, X., Tretyakova, I., Hidajat, R., Pulit-Penaloza, J. A., Belser, J. A., … Tumpey, T. M. (2016). Mono- and quadri-subtype virus-like particles (VLPs) containing H10 subtype elicit protective immunity to H10 influenza in a ferret challenge model. *Vaccine*, *34*(44), 5235–5242. https://doi.org/10.1016/j.vaccine.2016.09.012

Quan, F.-S., Kim, Y.-C., Song, J.-M., Hwang, H. S., Compans, R. W., Prausnitz, M. R., & Kang, S. -M. (2013). Long-term protective immunity from an influenza virus-like particle vaccine administered with a microneedle patch. *Clinical and Vaccine Immunology*, *20*(9), 1433–1439.

Quan, F. S., Kim, Y. C., Vunnava, A., Yoo, D. G., Song, J. M., Prausnitz, M. R., … Kang, S. M. (2010). Intradermal vaccination with influenza virus-like particles by using microneedles induces protection superior to that with intramuscular immunization. *Journal of Virology*, *84*(15), 7760–7769. https://doi.org/10.1128/jvi.01849-09

Ren, Z., Zhao, Y., Liu, J., Ji, X., Meng, L., Wang, T., … Xia, X. (2018). Intramuscular and intranasal immunization with an H7N9 influenza virus-like particle vaccine protects mice against lethal influenza virus challenge. *International Immunopharmacology*, *58*, 109–116.

Rezaei, F., Mirshafiey, A., Shahmahmoodi, S., Shoja, Z., Ghavami, N., & Mokhtari-Azad, T. (2013). Influenza virus-like particle containing two different subtypes of hemagglutinin confers protection in mice against lethal challenge with A/PR8 (H1N1) and A/HK (H3N2) viruses. *Iranian Red Crescent Medical Journal*, *15*(1), 75–82. https://doi.org/10.5812/ircmj.6252

Roldão, A., Mellado, M. C. M., Castilho, L. R., Carrondo, M. J., & Alves, P. M. (2010). Virus-like particles in vaccine development. *Expert Review of Vaccines*, *9*(10), 1149–1176. https://doi.org/10.1586/erv.10.115

Shim, J., Kim, J., Tenson, T., Min, J.-Y., & Kainov, D. (2017). Influenza virus infection, interferon response, viral counter-response, and apoptosis. *Viruses*, *9*(8), 223.

Si, Y., Wen, Y., Kelly, S. H., Chong, A. S., & Collier, J. H. (2018). Intranasal delivery of adjuvant-free peptide nanofibers elicits resident $CD8^+$ T cell responses. *Journal of Controlled Release*, *282*, 120–130.

Song, J. M., Hossain, J., Yoo, D. G., Lipatov, A. S., Davis, C. T., Quan, F. S., … Kang, S. M. (2010). Protective immunity against H5N1 influenza virus

by a single dose vaccination with virus-like particles. *Virology*, *405*(1), 165–175. https://doi.org/10.1016/j.virol.2010.05.034

Song, J.-M., Wang, B.-Z., Park, K.-M., Van Rooijen, N., Quan, F.-S., Kim, M.-C., … Kang, S. M. (2011). Influenza virus-like particles containing M2 induce broadly cross protective immunity. *PLoS One*, *6*(1), e14538.

Sung, S. S. J., Fu, S. M., Rose, C. E., Jr., Gaskin, F., Ju, S. T., & Beaty, S. R. (2006). A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing langerin and tight junction proteins. *Journal of Immunology*, *176*(4), 2161–2172.

Trondsen, M., Aqrawi, L. A., Zhou, F., Pedersen, G., Trieu, M. C., Zhou, P., & Cox, R. J. (2015). Induction of local secretory IgA and multifunctional $CD4^+$ T-helper cells following intranasal immunization with a H5N1 whole inactivated influenza virus vaccine in BALB/c mice. *Scandinavian Journal of Immunology*, *81*(5), 305–317. https://doi.org/10.1111/sji.12288

Wang, B. Z., Gill, H. S., Kang, S. M., Wang, L., Wang, Y. C., Vassilieva, E. V., & Compans, R. W. (2012). Enhanced influenza virus-like particle vaccines containing the extracellular domain of matrix protein 2 and a toll-like receptor ligand. *Clinical and Vaccine Immunology*, *19*(8), 1119–1125. https://doi.org/10.1128/cvi.00153-12

Wei, H. J., Chang, W., Lin, S. C., Liu, W. C., Chang, D. K., Chong, P., & Wu, S. C. (2011). Fabrication of influenza virus-like particles using M2 fusion proteins for imaging single viruses and designing vaccines. *Vaccine*, *29*(41), 7163–7172. https://doi.org/10.1016/j.vaccine.2011.05.077

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